Volume 234, number 1, 107–110 FEB 06009 July 1988

Direct electrochemistry in the characterisation of redox proteins: novel properties of *Azotobacter* 7Fe ferredoxin

Fraser A. Armstrong, Simon J. George*, Andrew J. Thomson* and M. Geoffrey Yates[+]

*Inorganic Chemistry Laboratory, Oxford University, South Parks Road, Oxford OX1 3QR, *School of Chemical Sciences, University of East Anglia, Norwich NR4 7TJ* and [+] *AFRC IPSR Nitrogen Fixation Laboratory, University of Sussex, Brighton BN1 9QJ, England*

Received 4 May 1988

Fast diffusion-dominated electron transfer between *Azotobacter chroococcum* 7Fe ferredoxin, FdI, and pyrolytic graphite 'edge' electrodes, promoted by aminoglycosides, permits detailed voltammetric studies and preparation of oxidation states inaccessible by chemical titration. The [3Fe-4S] cluster exhibits pH dependent $E_{1/2}$ values (30°C); $E_{1/2}$ (alkaline) = 460 ± 10 mV vs NHE, $-dE_{1/2}/d(pH) = 55$ mV, $pK = 7.8$. The [4Fe-4S] cluster is characterised by an unusually low reduction potential, −645 ± 10 mV vs NHE, at pH 8.3, with a slight pH dependence, $-dE_{1/2}/d(pH) \sim 25$ mV over the pH range 8.5–7.0. No redox couple is observed at potentials between −300 and +600 mV vs NHE. This shows that the [4Fe-4S] cluster is not an HIPIP-type centre. The electron paramagnetic resonance spectrum, centred at $g = 1.93$, of the product resulting from bulk electrolysis at −835 mV is assigned to a $[4Fe\text{-}4S]^{+}$ cluster interacting magnetically with a reduced [3Fe-4S] cluster.

Electrochemistry; Iron-sulfur cluster; Ferredoxin; (*Azotobacter*)

1. INTRODUCTION

Azotobacter contain a ferredoxin (FdI) which, when isolated aerobically from *Azotobacter chroococcum* [1] and *Azotobacter vinelandii* [2] contains two iron-sulphur clusters. One has a core structure [4Fe-4S] and the other comprises only three iron atoms [3]. In spite of extensive spectroscopic and crystallographic data on *A. vinelandii* FdI, the structure of the three-iron cluster has been unclear. The original X-ray analysis [4,5] is now known to be in error. From independent determinations of the structure [6,7] it is agreed that the three-iron cluster has a core structure [3Fe-4S] and is ligated by three cysteine ligands, residues 8, 11 and 16.

In a wider context, spectroscopic comparisons of oxidised forms of *Azotobacter* FdI, *Desulphovibrio gigas* FdII, and the inactive form of beef heart aconitase, using Mössbauer ([11] and references therein), magnetic circular dichroism (MCD) [8–10] and resonance Raman [12] show that a similar core structure must be present in each.

The redox properties of FdI have also been controversial. It was originally proposed that the four-iron cluster cycles between the oxidation states $[4Fe\text{-}4S]^{3+}$ and $[4Fe\text{-}4S]^{2+}$ [13]. This view was shown to be erroneous by Morgan et al. [14] who produced evidence that oxidation of this cluster with $K_3Fe(CN)_6$ leads to the partial destruction of the cluster and the production of an unusual thiol radical. They further showed that, in the presence of sodium dithionite above pH 8.0, the protein can be partially reduced to an electron paramagnetic resonance (EPR) active state [15]. This led them to propose that the cluster cycles $[4Fe\text{-}4S]^{2+}/[4Fe\text{-}4S]^{1+}$ at a very low redox potential. The three-iron cluster undergoes one-electron reduction at a reported potential of ~ −420 mV [13]. However,

Correspondence address: F.A. Armstrong, Inorganic Chemistry Laboratory, Oxford University, South Parks Road, Oxford OX1 3QR, England

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

recent MCD measurement on the three-iron cluster in *A. chroococcum* FdI has shown an interesting pH equilibrium between two forms of the reduced cluster, $[3Fe\text{-}4S]^0$ [8,9]. This equilibrium is also present, apparently, in *A. vinelandii* FdI [16].

In this paper we describe new investigations, exploiting direct (unmediated) electrochemical methods, of the [4Fe-4S] and [3Fe-4S] clusters of *A. chroococcum* FdI. In the presence of the aminoglycosides, tobramycin or neomycin, which promote direct interaction of the protein at pyrolytic graphite 'edge' (PGE) electrodes [17,18], *A. chroococcum* FdI gives fast heterogeneous electron transfer. This has allowed simultaneous determination, by cyclic voltammetry of the reduction potentials of both clusters in the protein, and has enabled us to study the pH dependence of these potentials over the range pH 6–9. Reduction of the three-iron cluster is linked to the process of proton uptake. Furthermore we have been able to carry out bulk electrolytic reduction of the protein at potentials much lower than the capability of sodium dithionite.

2. MATERIALS AND METHODS

Ferredoxin I was isolated from *A. chroococcum* by a modified literature procedure [2]. The protein used in this work had an absorbance index, A_{400}/A_{280}, of 0.58. It gave a single band on silver-stained SDS polyacrylamide gels. Samples for electrochemistry were prepared by dialysis against the required buffer-electrolyte solution in an Amicon ultrafiltration unit using a YM5 membrane. Concentrations were determined using $\epsilon_{400} = 29.8\ mM^{-1} \cdot cm^{-1}$ [14]. All chemicals used were of at least Analar grade.

Electrochemical measurements were made using an Oxford Electrodes potentiostat. DC cyclic voltammetry at various pH values was carried out with the protein solution, typically 50–100 μM FdI, contained in a thermostatted all-glass cell (temperature 3°C) with a three-electrode configuration. The PGE electrode was polished between measurements with a 0.3 μM alumina slurry and cleaned by sonication. Other details have been described [17,18]. Neomycin, tobramycin (each purchased from Sigma) or $Cr(NH_3)_6^{3+}$ were added as aliquots from concentrated stock solutions to promote electrochemical response of the protein. Adjustments of pH were made by small additions of concentrated NaOH or HCl to the sample solution which was buffered by a mixture of Pipes, Hepes and Taps (each 3.3 mM) in 0.1 M NaCl. Vigorous stirring by 'microflea' ensured rapid equilibrium. The resultant pH was measured with an MI-410 pH electrode (Microelectrodes Inc.).

Bulk electrolysis and confirmatory DC cyclic voltammetry were carried out using a capped, jacketed cell, capacity 400–500 μl, contained within an anaerobic glove box (O_2 < 1 ppm). The PGE working electrode formed the base of the cell sample compartment into which was positioned a reference side arm (Luggin tip) and an auxiliary electrode side arm with electrical contact maintained via a porous glass frit. Efficient stirring was achieved using a microflea. A full description of this apparatus will appear elsewhere. Following electrolysis, typically at 3°C, samples were withdrawn by syringe and transferred to EPR tubes.

EPR spectra were recorded on an X-band Bruker ER-200D spectrometer fitted with an Oxford Instrument ESR-900 flow cryostat. Spin integrations were performed using 1 mM Cu-EDTA as a standard.

3. RESULTS AND DISCUSSION

Fig.1 shows the cyclic voltammetry of *A. chroococcum* FdI in the presence of the aminoglycosides, tobramycin and neomycin. Well defined waves are observed.

Couple 'A' is pH dependent, quasi-reversible and diffusion dominated (at least to 100 $mV \cdot s^{-1}$) throughout. Use of $Cr(NH_3)_6^{3+}$ as a promoter enables the pH range of measurement to be extended above pH 8.5. With a requirement in excess of 5 mM, this is a much weaker promoter than the aminoglycosides for which 0.5–1.5 mM levels are adequate. Furthermore, the onset of $Cr(NH_3)_6^{3+}$ reduction below −750 mV limits its utility. However, unlike tobramycin and neomycin which deprotonate and become ineffective above pH 8.5, $Cr(NH_3)_6^{3+}$ remains an active promoter at least up to pH 9.4. At pH 6.3 neomycin (but not tobramycin) was found to cause precipitation.

The parameters obtained from fig.1 are $E_{1/2}$(alkaline) = −460 ± 10 mV, ΔEp (scan rate 10 $mV \cdot s^{-1}$, pH 8.3) = 80 mV, $dE_{1/2}$ (acid)/d(pH) = −55 mV (i.e. one H^+ per electron) and pK = 7.8 ± 0.1. We assign 'A' to the dithionite-reducible [3Fe-4S] cluster. The relevant equilibria are therefore:

$$[3Fe\text{-}4S]^{1+} + e^- \rightleftharpoons [3Fe\text{-}4S]^0 \quad (1)$$

$$[3Fe\text{-}4S]^0 + H^+ \rightleftharpoons [3Fe\text{-}4S]^0 \ldots H^+ \quad (2)$$

The marked contrast between the MCD spectra from the alkaline and the acidic forms indicates that protonation occurs at the chromophore or is linked to cluster re-organisation [8,9].

Couple 'B' is of similar intensity to 'A' and is defined clearly at pH > 7.0. At pH 8.3, promotion by tobramycin or neomycin yields excellent quasi-

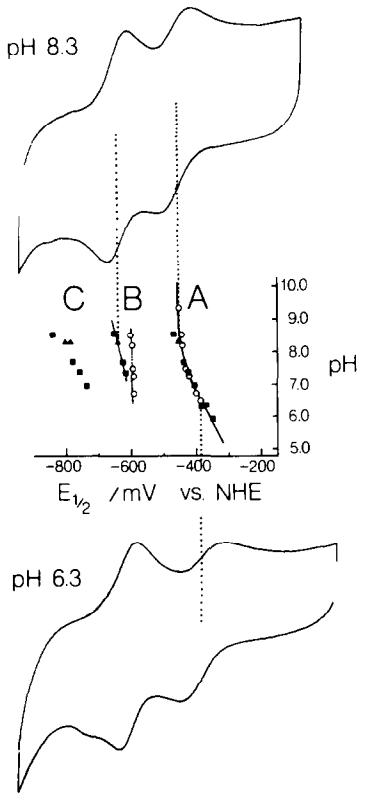

Fig.1. Cyclic voltammetry of *A. chroococcum* FdI at pH 8.3 (84 μM protein in 20 mM Taps, 0.10 M NaCl and 1.5 mM neomycin) and pH 6.3 (85 μM protein in 20 mM Pipes, 0.10 M NaCl and 1 mM tobramycin). Voltammograms were recorded at 3°C with scan rate 10 mV·s^{-1}. Shown at the centre is the pH dependence of $E_{1/2}$ values, for which solid lines correspond to: (A) $E_{1/2}$(alkaline) = −460 mV, $dE_{1/2}$(acid)/d(pH) = −55 mV, pK_{red} = 7.8; (B) $E_{1/2}$(pH 8.3) = −645 mV, $dE_{1/2}$/d(pH) = −25 mV. The pH was varied by additions to the protein (in 3.3 mM Pipes/3.3 mM Hepes/3.3 mM Taps mixed buffer, 0.10 M NaCl) of HCl or NaOH, with monitoring by an MI-410 pH micro-electrode. Promoting conditions are: (▲) neomycin, 1–2 mM; (■) tobramycin, 1–3 mM; (○) $Cr(NH_3)_6^{3+}$, 8 mM (for which the 'B' potential variation is indicated by closely spaced dots). Average $E_{1/2}$ values for 'C' are given but their accuracy is limited by low amplitude at high pH and mergence with 'B' at low pH.

reversible waves, ΔEp (low scan rate limit) = 55 mV, $E_{1/2}$ = −645 ± 10 mV and $dE_{1/2}$/d(pH) = −25 mV, which is diffusion dominated up to 1 V·s^{-1}. Alternatively, use of $Cr(NH_3)_6^{3+}$ yields $E_{1/2}$ = −600 ± 10 mV which is virtually independent of pH. This increase (cf. couple 'A') suggests binding of $Cr(NH_3)_6^{3+}$ close to the redox centre. Below pH 7.0 a quasi-reversible pH-dependent couple 'C' merges to interfere with 'B'. At pH 8.3, this is a minor feature with $E_{1/2} \simeq -800$ mV, yet upon acidification, anodic and cathodic waves are intensified. On prolonged scanning or reductive electrolysis 'C' disappears. The origins of this very low potential couple await further studies. One interesting possibility is that 'C' corresponds to reduction of a sulphur entity associated with the 3Fe centre (see for example [19]).

Anaerobic bulk reduction, at −835 mV, of *A. chroococcum* FdI at pH 8.3 caused significant bleaching of the ferredoxin. Cyclic voltammetry of the product demonstrated full retention of couples 'A' and 'B'. The resulting X-band EPR spectrum (fig.2) is complex. We assign this signal to a reduced (S = ½) $[4Fe\text{-}4S]^{1+}$ centre, and hence attribute couple 'B' to a $[4Fe\text{-}4S]^{2+,1+}$ cluster. The complex form of this spectrum closely resembles that observed from the reduced [4Fe-4S] cluster in the 7Fe ferredoxin from *Thermus thermophilus* [20]. Multiple-frequency EPR studies on the *Thermus* signal give changes which are consistent with spin-spin interactions of the $[4Fe\text{-}4S]^{1+}$ centre with the reduced (S = 2) three-iron cluster. The complex structure of the *Azotobacter* spectrum probably arises from a similar interaction.

Spin integration of this signal yielded 0.79 ±

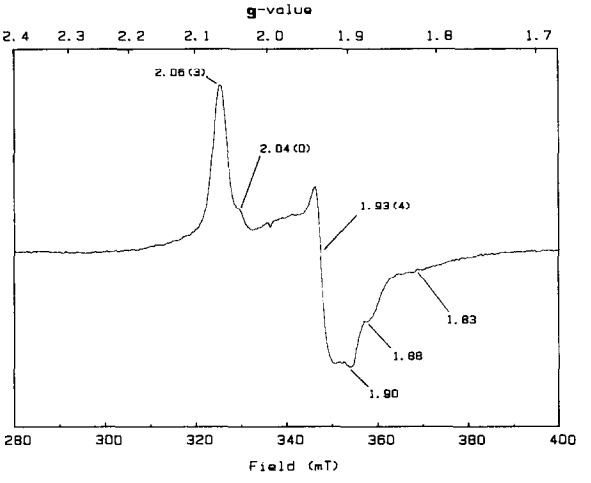

Fig.2. X-band EPR spectrum of *A. chroococcum* FdI (84 μM protein in 20 mM Taps, 0.10 M NaCl, and 1.5 mM neomycin at pH 8.3) electrochemically reduced at −835 mV. Temperature = 12 K, microwave power = 2 mW, microwave frequency = 9.40 GHz, modulation amplitude = 0.5 mT. Indicated on the spectrum are effective g-values.

0.10 spins per molecule, thereby indicating overall two-centre reduction by two electrons. For comparison, integration of the current-time decay for the reduction ($t_{1/2}$ = 20 min) gave 2.03 electron equivalents. With allowance for oxidation during sample transfer to an EPR tube this is a most satisfactory result.

In an analogous bulk reduction of a pH 6.3 solution, at −795 mV, we collected an EPR sample after the passage of 1.70 electron equivalents. The EPR spectrum was similar but not identical to that from the alkaline protein and showed spin-coupling interactions. Double integration yielded 0.57 ± 0.10 spins per molecule indicating overall protein reduction by 1.59 equivalents. However, in view of the complex overlay of redox activities observed at this pH, a satisfactory assessment awaits further studies.

Stephens et al. [15] have reported that dithionite reduction of *A. vinelandii* FdI at pH > 8.0 generates a time-dependent mixture of low-intensity EPR signals. They interpret their data in terms of an initial partial reduction of the [4Fe-4S] centre, followed by spontaneous conversion of the three-iron cluster to a four-iron cluster to yield an 8Fe Fd. The experiments presented here on *A. chroococcum* FdI show no time-dependent changes, the electrochemically reduced protein being unchanged after 2 h at 3°C. In addition, the EPR spectrum assigned by Stephens et al. to the reduced $[4Fe\text{-}4S]^{1+}$ cluster does not resemble that in fig.2. We are currently performing experiments designed to assess whether excess sodium dithionite may have a deleterious effect on *Azotobacter* ferredoxin.

We have been unable to find any redox couple at high potentials, i.e. up to 600 mV. This rules out entirely the original idea for *Azotobacter* Fd that the four-iron cluster is of the HIPIP type ($[4Fe\text{-}4S]^{3+,2+}$). On the contrary, our near quantitative generation of $[4Fe\text{-}4S]^{1+}$ without protein degradation confirms that this cluster is of the $[4Fe\text{-}4S]^{2+,1+}$ type, with an unusually low reduction potential. We believe that −645 mV represents, by a considerable margin, the lowest potential determined directly for a biological iron-sulphur cluster. With direct electrochemical methods we are now in a position to explore with ease and precision the 'high energy' redox chemistry that may lie beyond the range of abiotic reagents.

Acknowledgements: F.A.A. is grateful to the Royal Society for a University Fellowship and thanks Dr H.A.O. Hill for the provision of electrochemical facilities. A.J.T. and S.J.G. thank the SERC for grants in support of this work. M.G.Y. acknowledges funding from the AFRC.

REFERENCES

[1] Yates, M.G. (1970) FEBS Lett. 8, 281–285.
[2] Yoch, D.C. and Arnon, D.I. (1972) J. Biol. Chem. 250, 4515–4520.
[3] Emptage, M.H., Kent, T.A., Huynh, B.H., Rawlings, J., Orme-Johnson, W.H. and Münck, E. (1980) J. Biol. Chem. 1793–1796.
[4] Ghosh, D., Furey, W., O'Donnell, S. and Stout, C.D. (1981) J. Biol. Chem. 256, 4185–4192.
[5] Howard, J.B., Lorsbach, T.W., Ghosh, D., Melis, K. and Stout, C.D. (1983) J. Biol. Chem. 258, 508–522.
[6] Stout, G.H., Turley, S., Sieker, L.C. and Jensen, L.H. (1988) Proc. Natl. Acad. Sci. USA 85, 1020–1022.
[7] Stout, C.D. (1988) Proc. Natl. Acad. Sci. USA, submitted.
[8] George, S.J., Richards, A.J.M., Thomson, A.J. and Yates, M.G. (1984) Biochem. J. 224, 247–251.
[9] George, S.J. (1986) PhD Thesis, University of East Anglia, Norwich, England, pp.132–182 and pp.261–291.
[10] Johnson, M.K., Thomson, A.J., Richards, A.J.M., Peterson, J., Robinson, A.E., Ramsey, R.R. and Singer, T.P. (1984) J. Biol. Chem. 259, 2274–2282.
[11] Beinert, H. and Thomson, A.J. (1983) Arch. Biochem. Biophys. 222, 333–361.
[12] Johnson, M.K., Czernuszewicz, R.S., Spiro, T.G., Fee, J.A. and Sweeney, W.V. (1983) J. Am. Chem. Soc. 105, 6671–6678.
[13] Sweeney, W.V., Rabinowitz, J.C. and Yoch, D.C. (1975) J. Biol. Chem. 250, 7842–7847.
[14] Morgan, T.V., Stephens, P.J., Devlin, F., Stout, C.D., Melis, K.A. and Burgess, B.K. (1984) Proc. Natl. Acad. Sci. USA 81, 1931–1935.
[15] Stephens, P.J., Morgan, T.V., Stout, C.D. and Burgess, B.K. (1986) in: Frontiers of Bioinorganic Chemistry (Xavier, A.V. ed.) pp.637–646, VCH, Weinheim.
[16] Johnson, M.K., Bennett, D.E., Fee, J.A. and Sweeney, W.V. (1987) Biochim. Biophys. Acta 911, 81–94.
[17] Armstrong, F.A., Cox, P.A., Hill, H.A.O., Lowe, V.J. and Oliver, B.N. (1987) J. Electroanal. Chem. 217, 331–366.
[18] Armstrong, F.A. and Lannon, A.M. (1987) J. Am. Chem. Soc. 109, 7211–7212.
[19] Dryhurst, G., Kadish, K.M., Scheller, F. and Renneberg, R. (1982) in: Biological Electrochemistry, vol.1, pp.441–447, Academic Press, New York.
[20] Hagen, W.R., Dunham, W.R., Johnson, M.K. and Fee, J.A. (1985) Biochim. Biophys. Acta 828, 369–374.